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Propeptide of Human Protein C Is Necessary for γ-Carboxylation†

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ABSTRACT: Protein C is one of a family of vitamin K dependent proteins, including blood coagulation factors and bone proteins, that contains γ-carboxyglutamic acid. Sequence analysis of the cDNAs for these proteins has revealed the presence of a prepro leader sequence that contains a pre sequence or hydrophobic signal sequence and a propeptide containing a number of highly conserved amino acids. The pre region is removed from the growing polypeptide chain by signal peptidase, while the pro region is subsequently removed from the protein prior to secretion. In the present study, deletion mutants have been constructed in the propeptide region of the cDNA for human protein C, and the cDNAs were then expressed in mammalian cell culture. These deletions included the removal of 4, 9, 12, 15, 16, or 17 amino acids comprising the carboxyl end of the leader sequence of 42 amino acids. The mutant proteins were then examined by Western blotting, barium citrate adsorption and precipitation, amino acid sequence analysis, and biological activity and compared with the native protein present in normal plasma. These experiments have shown that protein C is readily synthesized in mammalian cell cultures, processed, and secreted as a two-chain molecule with biological activity. Furthermore, the pre portion or signal sequence in human protein C is 18 amino acids in length, and the pro portion of the leader sequence is 24 amino acids in length. Also, during biosynthesis and secretion, the amino-terminal region of the propeptide (residues from about −12 through −17) is important for γ-carboxylation of protein C, while the present data and those of others indicate that the carboxyl-terminal portion of the propeptide (residues −1 through −4) is important for the removal of the pro leader sequence by proteolytic processing.

Protein C is a precursor to a serine protease present in plasma and plays an important physiological role in the regulation of blood coagulation (Esmon, 1987). Protein C is a vitamin K dependent glycoprotein (M_r 62 000) composed of a heavy chain (M_r 41 000) and a light chain (M_r 21 000), and these two chains are held together by a disulfide bond (Stenflo, 1976; Kisiel et al., 1976). The light chain of human protein C contains nine residues of γ-carboxyglutamic acid (DiScipio & Davie, 1979; Fernlund & Stenflo, 1982) and one β-hydroxyaspartic acid residue (Drakenberg et al., 1983; McMullen et al., 1983). Protein C shows considerable amino acid sequence homology with the other vitamin K dependent plasma proteins involved in blood coagulation, including prothrombin, factor VII, factor IX, factor X, and protein S. Analyses of the cDNA (Foster & Davie, 1984; Beckman et al., 1985) and

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the gene (Foster et al., 1985; Plutzky et al., 1986) for human protein C have indicated that it is synthesized as a single-chain polypeptide which undergoes considerable processing to give rise to the two-chain molecule that circulates in blood.

Protein C, factor VII, factor IX, and factor X also share similarity in their domain organization. Each contains an amino-terminal γ-carboxylated domain, two epidermal growth factor like domains (Doolittle et al., 1984), a connecting region containing an activation peptide, and a trypsin-like serine protease domain. The structures of the genes for human protein C (Foster et al., 1985; Plutzky et al., 1986), factor VII (O'Hara et al., 1987), factor IX (Anson et al., 1984; Yoshitake et al., 1985), and factor X (Leytus et al., 1986) indicate that these genes are closely related, having introns that are highly conserved in their positions relative to their protein sequences.

Analysis of the cDNAs and genes for human prothrombin (Degen et al., 1983; Degen & Davie, 1986), factor VII (Hagen et al., 1986), factor IX (Kurachi & Davie, 1982; Jaye et al., 1983; Anson et al., 1984), factor X (Fung et al., 1985; Leytus et al., 1986), protein C (Foster et al., 1985; Beckman et al., 1985), and protein S (Lundwall et al., 1986; Hoskins et al., 1987) indicates that each also encodes a prepro leader sequence of 38–46 amino acids. Since the leader sequences end with basic amino acids that are poor cleavage sites for signal peptidase (Perlman & Halvorson, 1983; von Heijne, 1984), it has been proposed that these sequences are "prepro" peptides containing an N-terminal hydrophobic signal sequence, followed by a more highly charged propeptide of unknown function (Kurachi & Davie, 1982). The propeptide is removed from prothrombin, factor VII, factor IX, and the light chains of factor X and protein C during biosynthesis or secretion by a protease with a trypsin-like specificity. Alignment of the leader sequences for the vitamin K dependent proteins indicates the presence of several highly conserved or homologous amino acids in the apparent propeptide region in positions −1 through −17. The cDNA sequence for a γ-carboxylated protein from rat bone (osteocalcin) indicates that these residues are also conserved in the corresponding positions in the leader sequence for this protein (Pan & Price, 1985). The conservation of amino acids in the leader sequence, as well as the proximity of the leader sequence to the γ-carboxylated region, has led to the proposal that the propeptide may serve in part as a recognition signal for the γ-carboxylation complex associated with the endoplasmic reticulum (Pan & Price, 1985).

In order to test this hypothesis, a series of mutations have been constructed in the cDNA for human protein C that result in sequential deletions in the propeptide leader sequence. These constructions were then inserted into an expression vector and expressed in a γ-carboxylation-competent mammalian cell system. The mutant proteins that were secreted were then analyzed for γ-carboxylation, leader sequence processing, and biological activity. These experiments have identified the propeptide and have shown that it plays an important role in γ-carboxylation and processing of human protein C.

Materials and Methods

Materials. Purified human plasma protein C and affinity-purified polyclonal antibody prepared in sheep to human protein C were kindly provided by Dr. Walter Kisiel. Avidin-conjugated alkaline phosphatase was obtained from Boehringer Mannheim, and alkaline phosphatase substrate (Sigma 104) was from Sigma Chemical Co. "Protac C" protein C activator and the activated protein C amidolytic substrate H-D-Lys-(γ-Cbo)-Pro-Arg-pNA·AcOH (Spectrozyme PCa) were obtained from American Diagnostica, Inc., while CNBr-activated Sepharose was from Pharmacia.

Construction of the Expression Vector. A restriction fragment corresponding to exon 1 of the gene for human protein C (Foster et al., 1985) was nick-translated and used as a probe to screen a HepG2 cDNA library prepared in λgt11 (Hagen et al., 1986). A full-length cDNA was isolated which encoded 70 base pairs (bp) of the 5′ noncoding region, the entire coding sequence for human protein C, and the 3′ noncoding region corresponding to the second polyadenylation site (Foster & Davie, 1984; Beckmann et al., 1985). A 1760 bp insert coding for protein C was isolated from an *Eco*RI digest of the cDNA and ligated into the *Eco*RI site of the vector pDX to form pDX/PC. The pDX plasmid was derived from plasmid pD2 (Berkner & Sharp, 1985; Busby et al., 1985) and contained the adenovirus major late promoter (MLP), the cDNA encoding the entire tripartite leader (L), a splice set (SS) composed of the adenovirus 2 third leader 5′ splice site and an immunoglobulin 3′ splice site, the early SV40 polyadenylation signal (PA), and the SV40 enhancer (ENH). The *Bam*HI cloning site of pD2 was replaced with an *Eco*RI site.

Mutagenesis. A 330 bp *Eco*RI–*Sal*I fragment containing the 5′ noncoding region and the amino-terminal 86 amino acids of human protein C (42 amino acid prepropeptide and 44 amino acids of the light chain) was subcloned in a 3′ to 5′ orientation into M13mp11. Various mutations in this fragment were then prepared by the two-primer oligonucleotide-directed mutagenesis technique (Zoller & Smith, 1982). An ATG sequence in the 5′ noncoding region of the cDNA at nucleotide positions 8–10 represented a potential false translation initiation site. In order to direct all translation initiation to the ATG codon at position 71, the 5′ noncoding ATG sequence was removed by loop-out oligonucleotide mutagenesis with a primer hybridizing to nine nucleotides on either side of the 5′ noncoding ATG. Following the removal of the ATG codon starting at position 71, deletions in the leader sequence were performed on the modified template containing the same *Eco*RI–*Sal*I fragment. All deletion mutants were sequenced by the dideoxy chain termination technique to verify each mutation and the fidelity of the remaining sequence. RF DNA was prepared from mutant plaques, and the mutant *Eco*RI–*Sal*I fragments were used to replace the corresponding fragment of pDX/PC. This approach allowed direct sequence analysis of all DNA constructions which had been through the in vitro mutagenesis steps.

Transfections. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and vitamin K (5 μg/mL). Cells were cotransfected with the protein C mutant plasmids and pKO-neo (Southern & Berg, 1982) by the calcium phosphate procedure (Graham & van der Eb, 1973) or the DEAE-dextran technique (Sompayrac & Danna, 1981). For transient analysis, samples of the culture media were removed after 48–72 h following transfection. For protein analysis of transiently transfected cells, plates were switched to serum-free media consisting of Dulbecco's modified Eagle's medium containing insulin (5 μg/mL), fibronectin (2 μg/mL), transferrin (20 μg/mL), and vitamin K (5 μg/mL). For selection of stable colonies, the cells were divided 48 h after transfection into serum-containing media containing G418 (500 μg/mL). G418-resistant colonies were isolated after 14 days and grown individually for large-scale protein purification.

ELISA. Affinity-purified antibody to human protein C was added to each well of a 96-well microtiter plate (100 μL of 1 μg/mL in 0.1 M Na_2CO_3, pH 9.6) and incubated overnight at 4 °C. The wells were washed 3 times with 0.005 M phosphate buffer, pH 7.5, containing 0.15 M saline (PBS) and 0.05% Tween-20 and then incubated with 100 μL of 1% bovine

serum albumin and 0.05% Tween-20 in the same buffer overnight at 4 °C. The plates were rinsed several times with PBS, air-dried, and stored at 4 °C. To assay samples containing protein C, 100 μL of each sample was incubated for 1 h at 37 °C in the coated wells, and the wells were rinsed with 0.05% Tween-20 in PBS. The wells were then incubated for 1 h at 37 °C with a biotin-conjugated sheep polyclonal antibody to protein C (30 ng/mL) in PBS containing 1% bovine serum albumin and 0.05% Tween-20. The wells were rinsed with PBS and incubated again for 1 h at 37 °C with avidin conjugated to alkaline phosphatase in PBS containing 1% bovine serum albumin and 0.05% Tween-20. Wells were rinsed with PBS, and alkaline phosphatase activity was measured with 100 μL of Sigma 104 phosphatase substrate (600 μg/mL) in 10% diethanolamine, pH 9.8, containing 0.3 mM $MgCl_2$.

Activated Protein C Anticoagulant Activity Assays. Serum-free culture media from cells transfected with expression plasmids for human protein C were concentrated on an Amicon filter apparatus until the protein C concentration was 100–1000 ng/mL. The media were then dialyzed exhaustively against 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, containing 150 mM NaCl (TBS). Protein C in the dialyzed media was then activated by incubation at 37 °C for 1 h with 0.1 volume (1 unit/mL) of Protac C, a protein C activator from copperhead snake venom (Klein & Walker, 1986) obtained from American Diagnostica. Control experiments demonstrated that this volume of activator was sufficient to yield maximal amidolytic activity for protein C. Amidolytic activity was measured by adding 75-μL aliquots of the activation mixture to 10 μL of 200 mM ethylenediaminetetraacetic acid (EDTA) and 500 μL of 1 μM protein C substrate, H-D-Lys-(γ-Cbo)-Pro-Arg-pNA·AcOH, and measuring the change in A_{405} over 30 min. The anticoagulant activity of the activated protein C was assayed as described by Sugo et al. (1985). In this assay, 50 μL each of normal pooled human plasma, rabbit brain cephalin (10 mg/mL in TBS), and kaolin suspension (5 mg/mL in TBS) was mixed in a siliconized glass tube. After preincubation at 37 °C for 2 min, 100 μL of activated protein C diluted in TBS was added, and the incubation at 37 °C was continued for another 15 s. Clotting was then initiated by the addition of 50 μL of 25 mM $CaCl_2$ and the clotting time recorded.

Barium Citrate Adsorption and Precipitation. Sodium citrate (200 μL of 0.5 M) was added to 5-mL samples of each culture media and incubated on ice for 5 min. $BaCl_2$ (200 μL of 1.0 M) was then added, and the incubation was continued at 0 °C for another 30 min. The precipitate was collected by centrifugation at 3000 rpm for 2 min and washed by resuspending in 2.5 mL of cold PBS. It was then recentrifuged and washed by the same procedure. The final pellet was redissolved in 2 mL of 0.1 M trisodium citrate containing 0.1% bovine serum albumin. The redissolved sample and the supernatant were then dialyzed against PBS and assayed by ELISA as described.

Western Blot. Samples of concentrated and dialyzed serum-free culture media containing 50 ng of protein C as determined by ELISA were analyzed. One set of samples was reduced by the addition of 1% β-mercaptoethanol. The samples were denatured by boiling for 3 min, separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to nitrocellulose by standard methods. The Western blot was blocked by incubation in TBS containing 1% bovine serum albumin for 30 min and then incubated overnight in the same buffer containing 1×10^6 cpm/mL of iodinated anti-human protein C antibody (3×10^7 cpm/μg). The blot was washed in TBS containing 0.1% NP-40 for several hours and overexposed to X-ray film in order to visualize the light chains of the recombinant protein C. This was necessary since the polyclonal antibody recognized the light chain of protein C only weakly.

Affinity Purification of Recombinant Protein C. Culture media from clonal cell lines expressing protein C were collected, centrifuged to remove cell debris, and filtered through 0.45-μm filters prior to protein purification. In a typical affinity purification, 1 L of media containing 100–500 μg of protein C was passed through a 1.0 × 1.0 cm antibody column prepared by coupling 7 mg of polyclonal sheep antibody against human protein C to 2 g of CNBr-activated Sepharose 4B. Following media application, the column was washed with 100 mL of TBS, and the protein C was eluted with TBS containing 3 M KSCN. The eluted protein sample was dialyzed exhaustively against 50 mM NH_4HCO_3 and lypholized prior to amino acid sequence analysis.

Amino Acid Sequence Analysis. Automated Edman degradation was performed with an Applied Biosystems 470A sequencer. The phenylthiohydantoin (PTH)–amino acids were identified by high-performance liquid chromatography (HPLC) on a Dupont Zorbax-PTH column (0.46 × 25 cm) with an isocratic solvent system of 64% 6.7 mM H_3PO_4, pH 2.69, and 36% CH_3CN/tetrahydrofuran (THF) (18:16 v/v). The column was run at a flow rate of 1.2 mL/min at 37 °C and was monitored at 254 nm. The chromatography system included a Waters WISP-710B sample injector linked to a Varian VISTA-5500 liquid chromatograph and a VISTA-402 data analyzer.

RESULTS

In order to determine whether the propeptide region of human protein C might be involved in the vitamin K dependent carboxylation of the light chain of the molecule and proper proteolytic processing of the prepro leader sequence, a series of nucleotide deletions in the cDNA for human protein C were constructed in which portions of the propeptide were deleted by in vitro mutagenesis. These deletions begin at position −1 in the leader sequence and include the removal of 4, 9, 12, 15, 16, and 17 amino acids (Figure 1). All of the mutants have a deletion of the propeptide processing site starting at position −1. Since this residue has been shown to be critical for removal of the propeptide in human factor IX (Diuguid et al., 1986), it was expected that each mutant would have a hydrophobic pre sequence or signal sequence that would be removed by signal peptidase but would retain its propeptide (or a portion of its propeptide) at the amino terminus of the light chain. Thus, the residual light chain of protein C was anticipated to vary in length depending upon the size of the deletions and the number of residues of the original propeptide in the molecule.

For construction of the expression vectors, the native and mutant forms of the cDNA for human protein C were ligated into a mammalian cell expression vector comprised of the adenovirus major late promoter, the SV40 enhancer, the adenovirus tripartite leader sequence, and splice donor and splice acceptor sequences upstream of the cloning site and a polyadenylation site downstream, as well as the SV40 origin of replication (Figure 2). This readily permitted propagation of the vector in COS cells for expression. This vector has been employed previously for the expression of factor IX (Busby et al., 1985), factor VII (Berkner et al., 1986), GmCSF (Kaushansky et al., 1986), and erythropoietin (Powell et al., 1986) in a variety of different mammalian cell lines.

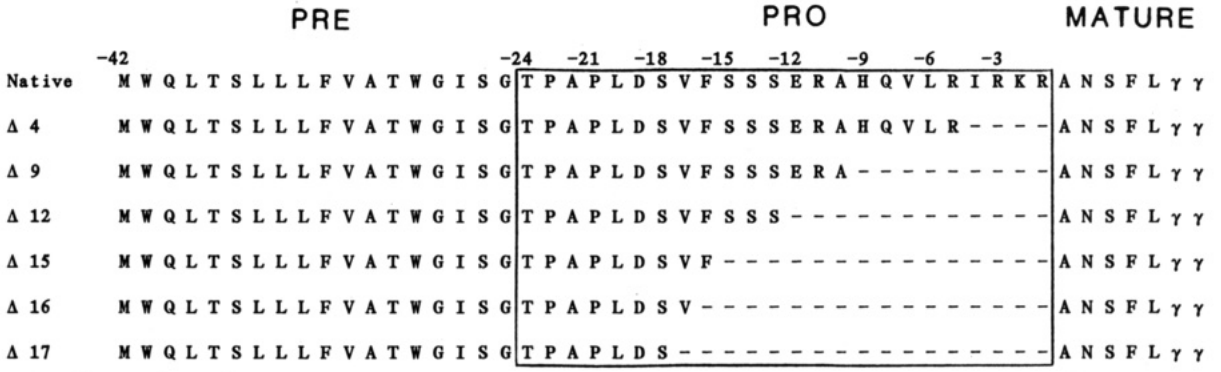

FIGURE 1: Leader sequences of human protein C deletion mutants created by site-directed mutagenesis. The pro portion of the leader sequence is boxed. The signal peptidase cleavage site occurs between the G and T at positions −25 and −24. Amino acid residues upstream from the mature amino terminus of the protein C light chain are identified with a negative number.

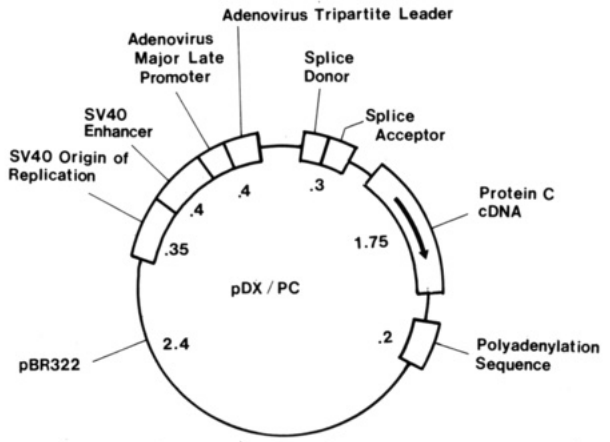

FIGURE 2: Mammalian cell expression vector used for expression of the human protein C mutants. The protein C cDNA was inserted into the pDX vector, as described under Materials and Methods.

Since the nucleotide deletions introduced into the cDNA for protein C were within the leader sequence, it was important to examine the potential effect of the deletion on the ability of mammalian cells to synthesize and secrete the mutant proteins. Accordingly, the plasmids for the Δ9 and Δ17 mutants and the native protein were transfected into monkey kidney COS cells by the calcium phosphate precipitation method, and 72 h after transfection, the culture media were collected and the cells harvested. An analysis for cellular protein C and protein C secreted into the culture medium is shown in Table I. These data indicated that approximately 90% of the recombinant protein C that was synthesized in the COS cells was secreted into the culture medium. This was true for the native recombinant protein as well as the 2 mutants that had deletions of 9 and 17 amino acids in their leader sequences. The signal sequence directing secretion from the cells was not affected by the deletions in the pro portion of the leader sequence. Although significant variations were observed in the transient expression level of protein C in different transfections, the percent of protein C that was secreted was relatively constant. In control experiments, it was shown that COS cells do not synthesize endogenous protein C.

In order to facilitate the analysis of a number of mutant proteins, human kidney cells and baby hamster kidney cells were compared with COS cells for their level of transient protein C production following transfection with the plasmid containing normal protein C. Culture media from these transfections were analyzed by an ELISA assay for protein C. These experiments indicated that human kidney cells secreted the highest levels of protein C and this level was consistently 5–10-fold higher than the two other cell lines tested.

Table I: Expression and Secretion of Recombinant Protein C from COS Cells[a]

plasmid	protein C in media (ng)	protein C in cell extract (ng)	protein C secreted (%)
none	0	0	
native protein C	165	20	89
Δ9 mutant	200	21	90
Δ17 mutant	80	12	87

[a] Culture media were collected from 10-cm plates 48 h after transfection with protein C expression plasmids. The cells were harvested with trypsin, washed twice with PBS, resuspended in PBS, and lysed by two cycles of freeze–thawing. Protein C in the culture media and in cell extracts was measured by ELISA.

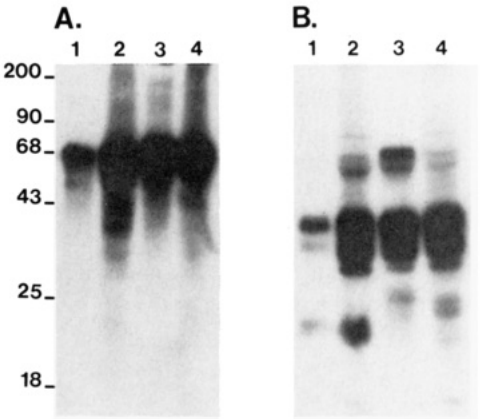

FIGURE 3: Western blot of native and mutant human protein C. Samples of culture media (or TBS buffer) containing 50 ng of each protein were run on 10% SDS–PAGE under nonreducing (panel A) or reducing conditions (panel B). Samples include purified native human plasma protein C (lane 1), native recombinant protein C (lane 2), the Δ9 deletion mutant (lane 3), and the Δ17 deletion mutant (lane 4). The gel was transferred and developed with ^{125}I-labeled polyclonal protein C antibody, as described under Materials and Methods.

Since human protein C is normally synthesized in the liver as a two-chain molecule, and since the deletions in the leader sequence were expected to affect the processing of the propeptide, the Δ9 and Δ17 mutant forms of protein C as well as the native protein were also analyzed for processing by Western blotting. In these experiments, the native recombinant protein C and two of the deletion mutants that were synthesized and secreted into the culture media of human kidney cells were subjected to SDS–polyacrylamide gel electrophoresis under both nonreducing and reducing conditions. Protein C was then identified with a radiolabeled polyclonal antibody

specific for human protein C. The three proteins studied (native, Δ9, and Δ17) had an apparent molecular weight of 68K under nonreducing conditions (Figure 3). This apparent molecular weight is identical with that of the plasma-derived human protein C. Reduction of the samples indicated that approximately 90% of each of the three different constructs yielded proteins that were processed into the two-chain form. This processing presumably occurs by the removal of the Lys-Arg dipeptide which connects the two chains of protein C after it is initially synthesized as a single chain (Foster & Davie, 1984). The reduced samples also revealed some heterogeneity in the size of the heavy chains of the recombinant proteins. All three samples had a heavy-chain band (M_r 42 000) which comigrated with the heavy chain of plasma protein C. They also had minor lower molecular weight bands of M_r 33 000–38 000. Since the heavy chain of human protein C contains three potential N-linked glycosylation sites (Foster & Davie, 1984), it is likely that these lower bands correspond to the heavy chains with reduced levels of glycosylation.

The light chain of the native recombinant protein C comigrated with that of the human plasma protein (M_r 22 000). The light chains of the deletion mutants lacking part (9 residues) or nearly all (17 residues) of the propeptide, however, had a higher molecular weight than that of the native molecule. This is consistent with the prediction that portions of the propeptides remained attached to the light chain of these molecules and were not removed during biosynthesis and secretion. As expected, the light chain from the −17 deletion mutant exhibited a lower molecular weight than that from the −9 deletion mutant (Figure 3). In control experiments, Western blot analysis indicated that the antibody was specific for human protein C and did not recognize bovine protein C which was present in the serum supplementing the culture medium.

Since the Western blot results suggested that the deletion mutants of protein C retained their propeptides, large quantities of the recombinant proteins were prepared for N-terminal amino acid sequence analysis to determine the site of signal peptidase cleavage and the presence or absence of γ-carboxyglutamic acid. Stable clonal cell lines synthesizing the protein C mutants were grown in large scale for production of approximately 500-mL quantities of media containing 500–1000 μg of the recombinant protein C. Protein C was purified by affinity chromatography on an anti-human protein C antibody–Sepharose column. After elution from the antibody column, samples of the affinity-purified proteins were subjected to automated Edman degradation. Since protein C is secreted as a two-chain molecule, two amino acids were identified in each cycle of the sequence analysis. Assignment of each amino acid to the light or heavy chains of protein C was straightforward since both sequences, as well as that of the leader sequence, were known (Foster & Davie, 1984; Foster et al., 1985; Beckmann et al., 1985).

The amino acid sequence for the first 13 residues of the light chain of native protein C as well as the yield at each cycle is shown in Figure 4 (panel A). The sequence of the native protein began with an alanine at position +1 in the light chain, indicating that the leader sequence had been correctly removed from the recombinant protein. No amino acid was identified at positions +6 and +7. These positions contain γ-carboxyglutamic acid in plasma protein C, and these residues were not extracted during the Edman degradation. Consequently, γ-carboxyglutamic acid was not detected by this sequence analysis. These data are consistent with the conclusion that the native recombinant protein C contains γ-carboxyglutamic

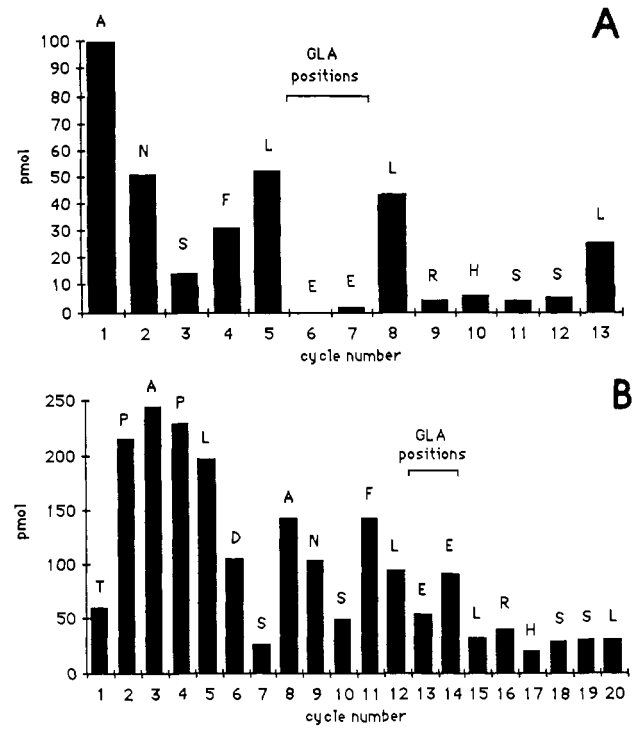

FIGURE 4: Amino acid sequence of recombinant protein C light chains. Samples of affinity-purified protein C (75 μg) were dialyzed against 50 mM NH_4HCO_3, lyophilized 4 times, and subjected to automated Edman degradation, as described under Materials and Methods. The light-chain sequences are native recombinant protein C (panel A) and the Δ17 propeptide deletion mutant (panel B).

acid in these positions rather than glutamic acid. The second sequence of DTED for the native recombinant protein C corresponds to the N-terminus of the heavy chain of the molecule (Kisiel, 1979). Thus, the recombinant protein C that is initially synthesized as a single-polypeptide chain is cleaved into the normal light and heavy chains during the processing and secretion of the protein.

The sequence of the light chain for both the Δ9 and Δ17 deletion mutants began with the sequence of TPAP. This sequence starts at position −24 in the prepro leader sequence of protein C. These experiments demonstrate that the carboxyl portion of the propeptide is required for proteolytic cleavage and removal of the leader sequence from human protein C. These data also show that the signal peptidase cleavage site in human protein C is between Gly at position −25 and Thr at position −24, a site that conforms to the "−1, −3" rule proposed for signal peptidase cleavage sites (Perlman & Halvorson, 1983; von Heijne, 1984). These experiments indicate that the leader peptide can be divided into a hydrophobic prepeptide of 18 amino acids (−42 through −25) and a more highly charged propeptide of 24 amino acids (−24 through −1).

The amino acid sequence of the light chain of the Δ17 deletion mutant was TPAPLDSANSFLEELRHSSL (Figure 4, panel B). This sequence is the same as the amino acid sequence of the pro leader sequence from positions −24 through −18 followed by the sequence of ANSFLEELRH. The last 10 residues correspond to the amino terminus of the mature light chain of protein C. Thus, the amino acid sequence confirms the deletion of DNA encoding residues −17 through −1. In cycles 13 and 14, glutamic acid was identified in good yield. These glutamic acid residues correspond to positions +6 and +7 of the light chain where the first two Gla residues are located in the native protein C. These data clearly demonstrate the absence of γ-carboxylation in the Δ17 mutant. These experiments are also consistent with the lack of ad-

Table II: Adsorption and Precipitation of Recombinant Protein C with Barium Citrate[a]

plasmid	% in barium citrate	
	supernatant	pellet
native protein C	30	70
Δ4 mutant	41	59
Δ9 mutant	60	40
Δ12 mutant	47	53
Δ15 mutant	74	26
Δ16 mutant	94	6
Δ17 mutant	94	6

[a] Human kidney cells were transfected with protein C expression plasmids as detailed under Materials and Methods. After 48 h, the culture media were collected, and the protein C was precipitated with barium citrate as described under Materials and Methods. Protein C present in the barium citrate supernatant and the redissolved pellet was measured by ELISA. The results presented are the average of three independent measurements.

sorption and precipitation of this protein with barium citrate, as described below.

The amino acid sequence of the light chain of the Δ9 deletion mutant matched the amino acid sequence of the leader sequence from positions −24 to −10 followed by the sequence of the light chain of protein C. These data also confirm the DNA deletion mutants. Cycles 21 and 22 of the amino acid sequence analysis correspond to positions +6 and +7 in the light chain of plasma protein C. No glutamic acid was detected in cycle 21, and only a small amount of glutamic acid was detected in cycle 22, as compared with the amino acids detected in cycles 19, 20, and 23. This is consistent with reduced, but significant, γ-carboxylation of the Δ9 mutant protein. These experiments show that carboxylation readily occurs in the native recombinant protein C and the Δ9 deletion mutant but carboxylation does not occur in the Δ17 deletion mutant when expressed in human kidney cells.

To further assess the effect of deletions in the leader sequence on the relative levels of γ-carboxylation of the protein C mutants, additional mutants with deletions of 4, 12, 15, and 16 residues as well as the Δ9 and Δ17 mutants were expressed in human kidney cells and analyzed by barium citrate adsorption and precipitation. This procedure has been shown to be capable of selectively adsorbing and precipitating only the γ-carboxylated proteins from plasma (Bajaj et al., 1981) as well as the carboxylated and active form of recombinant factor IX from a culture medium of baby hamster kidney cells (Busby et al., 1985). In these experiments, the protein C plasmids with each of the six deletions were introduced into cells by the calcium phosphate precipitation procedure, and samples from the culture medium were collected after 72 h. The recombinant proteins were then adsorbed and precipitated with barium citrate, and the concentration of protein C remaining in the culture medium and in the redissolved barium citrate pellets was determined by ELISA. Table II shows that approximately 70% of the native recombinant protein C was adsorbed to barium citrate. Furthermore, deletion of up to 12 amino acids from the carboxyl end of the pro leader peptide had only a small effect on barium citrate adsorption and precipitation, compared to deletions of 15–17 amino acids in the carboxyl region of the propeptide. These data further support the conclusion that the carboxyl-terminal 9–12 amino acids of the pro leader sequence have a relatively small effect on the carboxylation of protein C, while the remaining portion of the pro leader sequence (from about position −12 through −17) plays a major role in the carboxylation reaction.

The anticoagulant activities of the recombinant protein C as well as the Δ9 and Δ17 mutants were also measured to determine the effect of reduced γ-carboxylation and the presence of the propeptide on the biological properties of protein C. In order to measure the anticoagulant activity of protein C in the culture media, it was necessary to grow the cells in serum-free media to eliminate the procoagulant activity of bovine serum components. The cell culture media were then collected and dialyzed against Tris-buffered saline, and protein C was activated by incubation with Protac C, a specific copperhead venom derived protein C activator (Klein & Walker, 1986). When maximal amidolytic activity toward H-D-Lys-(γ-Cbo)-Pro-Arg-pNA·AcOH was obtained, the samples were assayed for their capacity to prolong the kaolin–cephalin clotting time of pooled, normal human plasma. These experiments demonstrate that the native recombinant protein C had normal anticoagulant activity, with a specific activity in the same range as human plasma protein C (Figure 5). The Δ9 and Δ17 deletion mutants, however, showed no anticoagulant activity, indicating that the presence of the propeptide or possibly the reduced γ-carboxylation (or both) interfered with normal anticoagulant activity of these two mutant proteins.

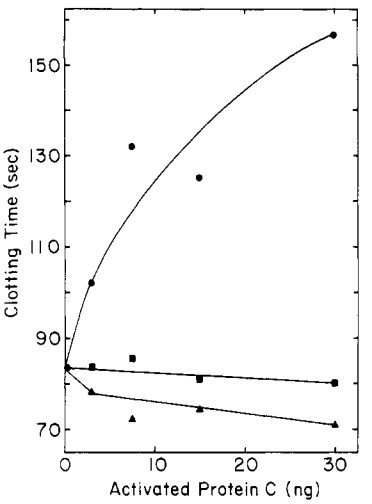

FIGURE 5: Anticoagulant activity of protein C. Serum-free culture media containing native recombinant or mutant protein C were dialyzed against TBS buffer, activated to maximal protein C amidolytic activity, and evaluated in a two-stage clotting assay for the ability to prolong the kaolin–cephalin clotting time of pooled normal human plasma, as described under Materials and Methods. (●) Native recombinant protein C; (■) Δ9 mutant; (▲) Δ17 mutant.

Discussion

Human protein C undergoes extensive co- and posttranslational modification in the liver to form the mature, two-chain protein circulating in plasma. These processing steps include removal of the hydrophobic prepeptide of 18 amino acids by signal peptidase, proteolytic removal of the more highly charged propeptide of 24 amino acids, γ-carboxylation of the first 9 glutamic acid residues near the amino terminus of the light chain, β-hydroxylation of aspartic acid-71 in the light chain, proteolytic removal of the Lys-Arg connecting dipeptide, and addition of carbohydrate to several N-linked glycosylation sites. The molecular signals which direct some of these modifications have been fairly well characterized. These include, for example, cleavage of the typical hydrophobic leader sequence by signal peptidase and addition of carbohydrate to Asn-X-Thr/Ser sequences. The amino acid sequences which signal these modifications are conserved within a large diversity of proteins. The molecular signals which direct the other modifications of the prepro protein C, such as the proteolytic processing, γ-carboxylation, and β-hydroxylation, are less well

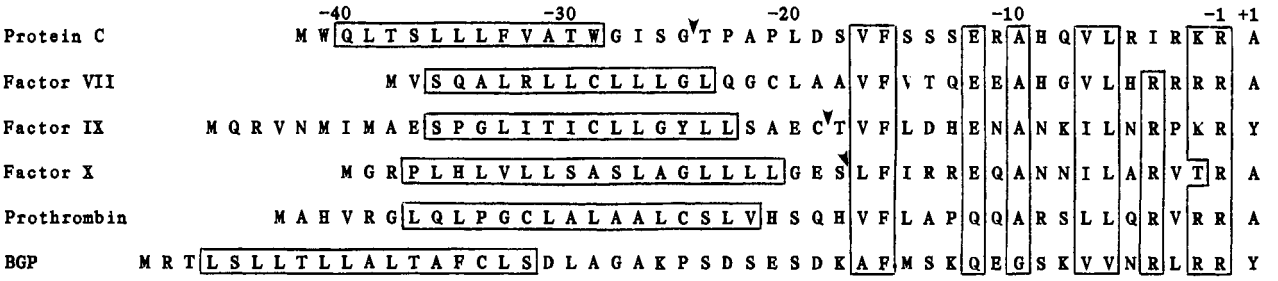

FIGURE 6: Leader sequence of γ-carboxylated proteins from human plasma and rat bone. The sequences are taken from published cDNA sequences for human protein C (Foster et al., 1985; Beckmann et al., 1985), factor X (Fung et al., 1985; Leytus et al., 1986), factor VII (Hagen et al., 1986), factor IX (Kurachi & Davie, 1982; Anson et al., 1984), prothrombin (Degen & Davie, 1986; MacGillivray et al., 1986), and rat bone Gla protein (BGP) (Pan & Price, 1985). The putative hydrophobic core of each signal sequence is boxed. The known signal peptidase cleavage sites for protein C (this work), factor X (by analogy to bovine factor X; Blanchard et al., 1985), and factor IX (Bentley et al., 1986; Diuguid et al., 1986) are indicated with arrowheads. Highly conserved and homologous amino acid residues within the propeptide region of the leader sequences are also boxed. Numbering is relative to the mature amino termini of the proteins.

characterized and understood.

One approach toward understanding these modification signals is to compare precursor proteins that are known to undergo very similar modifications. Such a structural comparison of the precursors of the human vitamin K dependent proteins has revealed that these proteins show extensive amino acid sequence and structural homologies with each other. This homology is particularly strong in the N-terminal regions of the vitamin K dependent proteins where the γ-carboxylated glutamic acid residues are located. Furthermore, strong amino acid sequence homology between these proteins extends into the propeptide region of the leader sequences, with portions of the sequence between −1 and −17 being strongly conserved (Figure 6). The pre region of the leader sequences does not retain sequence homology within this family of proteins, but each protein does contain a hydrophobic core of amino acids typical of signal peptides.

The leader sequence of bovine factor X has been reported to be cleaved by signal peptidase between Ser and Val at positions −18 and −17, resulting in a prepeptide of 23 amino acids and a propeptide of 17 amino acids (Blanchard et al., 1985). Similarly, the precursor to human factor IX has been shown to be cleaved between the Glu and Cys at positions −19 and −18, resulting in a prepeptide of 28 amino acids and a propeptide of 18 amino acids (Bentley et al., 1986; Diuguid et al., 1986). Thus, it is appropriate to view these leader sequences in the vitamin K dependent proteins as prepro leader sequences, as originally proposed (Kurachi & Davie, 1982), with an N-terminal hydrophobic signal peptide of approximately 18–28 amino acids and a C-terminal propeptide of approximately 17–24 amino acids. The amino acid sequence conserved in portions of the propeptides, as well as the proximity of the propeptide to the γ-carboxylated region, has led to the proposal that the propeptide may be recognized by the vitamin K dependent carboxylase which participates in the γ-carboxylation reaction (Pan & Price, 1985).

In the present experiments, mutations were introduced into the cDNA of human protein C by in vitro mutagenesis in order to create deletions of various portions of the propeptide between positions −1 and −17. The effect of the deletions on the level of γ-carboxylation was measured by expression of the mutant cDNA in a mammalian cell line that was carboxylation competent, followed by analysis of the mutant proteins by N-terminal sequence analysis, biological activity, and adsorption and precipitation with barium citrate.

Deletions in the propeptide region did not impair secretion from the cells (Table I). This is consistent with the existence of a separate secretion signal in the N-terminal portion of the leader seuence. Sequence analysis of the protein C mutant proteins showed that all of the deletion mutants began with the sequence of Thr-Pro-Ala-Pro- in their light chains. This indicates that the signal peptidase cleavage site is between the amino acids at positions −25 and −24 and permits designation of a signal sequence of 18 amino acids (from −42 through −25) and a propeptide of 24 amino acids (from −24 through −1) in human protein C.

An amino acid sequence analysis of the light chain of the native recombinant protein C indicated that the propeptide was removed by a protease with trypsin-like specificity. The requirement for the basic residues for propeptide cleavage was consistent with the observation that the deletion mutants which were missing these amino acids retained their propeptides at the N-terminus of the light chain. This is also consistent with the observation that an Arg in position −1 is necessary for cleavage of the propeptide from human factor IX and the formation of a biologically active molecule (Diuguid et al., 1986).

The sequence of the heavy chain for native recombinant protein C as well as the mutants began with Asp-Thr-Glu-Asp. This is identical with the sequence of the heavy chain for human plasma protein C. These data show that the recombinant protein C was cleaved into two chains following the Lys-Arg connecting dipeptide. It is not known, however, whether the Lys-Arg was removed by subsequent proteolysis from the C-terminal end of the light chain during its biosynthesis and secretion in cell culture. Analysis of the recombinant protein C (native and mutants) under reducing conditions on polyacrylamide gel electrophoresis indicated that approximately 90–95% of the protein had been cleaved into two chains by cleavage at this site (Figure 3). This processing step was considerably less efficient when recombinant protein C was expressed in other mammalian cell lines, indicating considerable variation in the processing by the protease(s) responsible for the cleavage.

The deletions in the propeptide region showed a pronounced effect on the level of γ-carboxylation of the mutant proteins. The degree of barium citrate precipitation decreased from approximately 70% for the native protein C to 5% in the Δ17 mutant, missing all of the region with high homology to the propeptides of other γ-carboxylated proteins. Intermediate deletions which retained portions of the highly homologous region showed intermediate levels of barium citrate adsorption and precipitability.

The most strongly conserved amino acids in the propeptide are Val-Phe at positions −17 and −16, Glu or Gln at −12, Ala at −10, Val-Leu at −7 and −6, Arg at −4, and Lys-Arg at −2 and −1 (Figure 6). Of these residues, the basic amino acids at −4, −2, and −1 probably serve as a signal for proteolytic

cleavage of the propeptide. The Val-Leu at positions −7 and −6, and the Ala at −10, although strongly conserved, do not appear to be critical for interaction with the carboxylation machinery for protein C since deletions up to −12 residues still resulted in significant γ-carboxylation. The Val and Phe in positions −17 and −16 in the propeptide, however, appear to be very critical for recognition by the carboxylaton complex, since deletion of these residues causes a precipitous decrease in the adsorption and precipitation of the protein by barium citrate and loss of γ-carboxylation as determined by amino acid sequence analysis. Both of these amino acids are conserved in all the propeptide sequences in the vitamin K dependent coagulation factors, and the Phe at −16 is also conserved in the bone Gla protein precursor (Figure 6). Taken together, these data suggest a critical role for Val-Phe at positions −17 and −16 in signaling recognition of these precursor proteins by the cellular γ-carboxylation machinery.

These data are consistent in part with two other recent reports published during the preparation of this paper. These reports have also implicated the propeptides of factor IX (Jorgensen et al., 1987) and protein C (Suttie et al., 1987) for recognition by the γ-carboxylation complex. However, the present data differ significantly from each of these reports. Jorgensen and co-workers found that either deletion of the propeptide or mutation of the Phe at −16 completely abolished γ-carboxylation of factor IX. These results are in complete agrement with the present data on human protein C. However, the experiments with factor IX also indicate that a mutation of the Ala at −10 in the propeptide completely abolishes γ-carboxylation. In the present studies, deletion of the carboxyl-terminal 12 residues of the propeptide of protein C, which includes the Ala at −10, had a relatively small effect on the γ-carboxylation of protein C, compared to deletions of 15, 16, and 17 amino acids in the propeptide. The reason for the difference between these two proteins is not obvious.

Suttie and co-workers (Suttie et al., 1987) found that a protein C precursor containing a partial propeptide of residues −1 through −10 supported γ-carboxylation in vitro more than a precursor without a propeptide. These data suggest an important role for the residues between −1 and −10 in the recognition by the γ-carboxylation complex. The reasons for the discrepancy between the present data and that of Suttie and co-workers are not obvious but may be due in part to a major difference in the relative rates of γ-carboxylation for their protein C substrate compared to those studied in the present investigation.

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Binding Properties of Solubilized Gonadotropin-Releasing Hormone Receptor: Role of Carboxylic Groups†

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ABSTRACT: The interaction of ^{125}I-buserelin, a superactive agonist of gonadotropin-releasing hormone (GnRH), with solubilized GnRH receptor was studied. The highest specific binding of ^{125}I-buserelin to solubilized GnRH receptor is evident at 4 °C, and equilibrium is reached after 2 h of incubation. The soluble receptor retained 100% of the original binding activity when kept at 4 or 22 °C for 60 min. Mono- and divalent cations inhibited, in a concentration-dependent manner, the binding of ^{125}I-buserelin to solubilized GnRH receptor. Monovalent cations require higher concentrations than divalent cations to inhibit the binding. Since the order of potency within the divalent cations was identical with that of their association constants to dicarboxylic compounds, it is suggested that there are at least two carboxylic groups of the receptor that participate in the binding of the hormone. The carboxyl groups of sialic acid residues are not absolutely required for GnRH binding since the binding of ^{125}I-buserelin to solubilized GnRH receptor was only slightly affected by pretreatment with neuraminidase and wheat germ agglutinin. The finding that polylysines stimulate luteinizing hormone (LH) release from pituitary cell cultures with the same efficacy as GnRH suggests that simple charge interactions can induce LH release. According to these results, we propose that the driving force for the formation of the hormone–receptor complex is an ionic interaction between the positively charged amino acid arginine in position 8 and the carboxyl groups in the binding site.

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH,[1] pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) is the primary regulator of the reproductive cycle. Binding of this hormone to specific receptors on pituitary gonadotrope cell membranes initiates the processes that lead to gonadotropin release. Characterization of the GnRH receptor in the pituitary has indicated that the receptor is a sialoglycoprotein with an apparent M_r of 60K (Hazum, 1981a,b, 1982; Schvartz & Hazum, 1985). Furthermore, using specific chemical reagents, we have identified two aromatic amino acid residues and two carboxylic groups in the membrane-associated GnRH receptor that are likely to be essential for the formation of the hormone–receptor complex (Keinan & Hazum, 1985).

Recently, we have succeeded in solubilizing the GnRH receptor from rat and bovine pituitary membrane preparations in an active form (Hazum et al., 1986, 1987) by using the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). In the present study, we report the characterization of the binding properties of the solubilized GnRH receptor, as well as the role of carboxylic groups of the receptor in both GnRH binding and the biological activity.

Materials and Methods

Materials

CHAPS and neuraminidase type V were purchased from Sigma, Dextran T-70 from Pharmacia, charcoal (Norit A) was from Fisher, wheat germ agglutinin (WGA) was from Miles Yeda, and Na^{125}I was from Amersham. [D-Ser(*t*-Bu)^6]des-Gly^{10}-GnRH ethylamide (buserelin) was kindly supplied by Dr. J. Sandow, Hoechst, Frankfurt. Polylysine and poly(glutamic acid) were generously supplied by I. Jakobson.

Methods

Iodination and Pituitary Membrane Preparations. [D-Ser(*t*-Bu)^6]des-Gly^{10}-GnRH ethylamide (buserelin) was iodinated by the lactoperoxidase method (Sandow & Konig, 1979). Specific activity of the labeled peptide was approximately 1.0 mCi/μg, as measured by self-displacement in the pituitary radioligand receptor assay. Pituitary membranes were prepared from 25–28-day-old Wistar-derived female rats according to Heber and Odell (1978), with modification. Briefly, the glands were homogenized gently with a tight Dounce homogenizer at 4 °C in 10 mM Tris·HCl, pH 7.4, and

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[1] Abbreviations: GnRH, gonadotropin-releasing hormone; buserelin, [D-Ser(*t*-Bu)^6]des-Gly^{10}-GnRH ethylamide; LH, luteinizing hormone; WGA, wheat germ agglutinin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PBS, phosphate-buffered saline.

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